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14. ABSTRACT
The goal of our research is to introduce a novel means to regenerate the articular cartilage and restore normal function of the joint. A strategy that can generate durable hyaline articular cartilage, which will be predominantly type II collagen, and is capable of integrating with the surrounding cartilage matrix (without fissures) could improve the long-term outcome of joint surface repair. Key findings are isolation of MSCs and stimulation towards osteogenesis in vitro on an octacalcium phosphate scaffold showing cells populated the scaffold and calcium deposits demonstrated with von Kossa stains; 2) A degradable form of photochemically crosslinked PEG norbomene gel was formulated and growth factors (TGF beta) tethered to the polymer showing peri-cellular cartilage matrix around the encapsulated swine chondrocytes; 3) Formation of new cartilage matrix was demonstrated in vivo in mice using photochemically crosslinked gels and swine articular chondrocytes 4) Chondrocytes encapsulated in photochemically crosslinked hydrogels can survive the crosslinking and implantation process as shown in swine 5) Bone marrow derived mesenchymal stem cells from swine were encapsulated in alginate, stimulated with chondrogenic growth factors, and showed in vivo matrix productions

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INTRODUCTION

Injuries to the cartilage surfaces of joint are particularly problematic because, unlike bone and other vascular tissues comprising the joint, cartilage is avascular and possesses limited capacity for repair and self-regeneration. Consequently, injury to cartilage in the articulating joints from trauma results in scar formation and possible arthritic changes that can lead to pain, stiffness, and loss of structure and function [1-3]. These joint injuries not only limit physical activity and mobility of those afflicted, but the inability to move freely can cause deep psychological scars and loss of independence when individuals have to depend on family and healthcare providers for constant assistance to perform daily life functions. The level of functional capability in the injured limb and ultimate quality of life depend on the successful outcome of joint surface regeneration performed as a secondary procedure weeks or even months after the initial injury. The return of function and the probability of return to active duty rely on successful restoration of the entire joint including the articular surface, and therefore, joint function. Lesions in the joint surface are commonly treated with microfracture [4], autologous cell implantation (ACI) [5], or osteoarticular autograft transfer system (OATS) [6]. To date, however, the outcomes of many restorative procedures are very unsatisfactory and an improved method for joint repair is a clear unmet need in military medicine. **The goal of our research is to introduce a novel means to regenerate the articular cartilage and restore normal function of the joint.** A strategy that can generate durable hyaline articular cartilage, which will be predominantly type II collagen, and is capable of integrating with the surrounding cartilage matrix (without fissures) could improve the long-term outcome of joint surface repair. *The scope of this research is to develop regenerative medicine approaches involving biocompatible hydrogel scaffolds seeded with autologous cells that provide three-dimensional environments favorable for promoting chondrogenesis for joint surface repair*.[7-10]

In the previous annual reports covering the first 24 months of this 48 month project, we reported on generating two candidate photochemically crosslinked hydrogels for encapsulating chondrocytes or chondrocyte precursor cells. Photochemically crosslinked gels were made using collagen as a natural protein gel and poly(ethylene) glycol thiol-ene gels as a synthetic gel. The photochemically crosslinked collagen gels demonstrated increased resistance to collagenase digestion over uncrosslinked gels, but had little effect on changing the bulk modulus (stiffness) of the gels. Work reported in the year 2 annual report showed the results of a preliminary study on cartilage formation using photochemically crosslinked collagen gels in vivo in mice. A modification to the gelation process involved performing the polymerization process in an hypoxic environment to improve cell viability. Testing of the PEG thiol-ene gels showed changes in the shear moduli were related to the weight percent of the gels. In vitro studies showed good viability of swine articular chondrocytes following the polymerization process. Work in year three and presented in this report builds upon the in vivo work with photochemically crosslinked collagen and fibrin gels showing cartilage formation in mice. Modifications of the PEG thiol-ene gels by linking growth factors to the polymer chains is reported and a manuscript has been submitted to the Journal of Biomedical Materials Research (JBMR) (Appendix C). Results in year 2 suggested that the size of the original defects (6.5 mm) was too large to permit satisfactory regeneration of the joint surface. A pilot study using collagen gels in a defect measuring 1mm in diameter in swine is reported showing new cartilage formation in the defects and filling the lesions in the articular cartilage.

This annual report covering the third 12 months of the project reviews our progress testing the physical and chemical properties of these photochemically crosslinked hydrogels and their ability to promote chondrogenesis. The tasks referenced below pertinent to Year 3 under Task 1 include subtasks 1.1.b, 1.1.c, 1.2.a, and under Task 2 include subtask 2.1.a.

BODY

Task 1 Test of photochemically crosslinked gels to produce cartilage and bone using chondrocytes and osteoblasts

Subtask 1.1.b Perform implantation of photochemically crosslinked collagen and PEG gels with chondrocytes and osteoblasts

- In vitro preparation and evaluation of bone matrix formation of cells seeded on scaffold.

Results: Octacalcium phosphate (OCP) has been reported to be a strong osteoinductive material and to produce more bone formation than other scaffolds, such as hydroxyapatite and β-tricalcium phosphate [11,12]. To make the OCP crystals, a 0.04M calcium acetate solution is slowly added to a 0.04M sodium monobasic phosphate solution over 1 hour. The precipitate is then sieved to 300 – 500μm size and sterilized prior to cell seeding [13]. Swine mesenchymal stem cells (MSCs) were seeded on a porous octacalcium phosphate scaffold. The swine MSCs were seeded at a density of $2x10^4$ cells/20mg of OCP crystals for 16 hours. Tissue culture plastic (TCP) was used as the control. A live/dead stain was performed after cell seeding to assess cell adhesion to crystals (Figure 2). The OCP and TCP groups were cultured using an osteogenic induction media and a maintenance media. After 2 weeks of incubation, a von kossa stain and hematoxylin and eosin stain was performed for each group (Figure 3). Despite the difficultly of imaging the OCP crystals, calcium deposits were seen for both the induction and maintenance media groups suggesting that the OCP crystals themselves induce osteogenesis. This experiment will be repeated and expanded in year 4 to quantify and optimize the seeding potential of OCP, to evaluate the osteoconductive and osteoinductive nature of OCP, and to evaluate bone matrix production in vivo.

- In vitro preparation and testing PEG gels for cytocompatibility and strength

Results: A poly(ethylene) glycol (PEG) hydrogel system was reported in year 2 to deliver cells to defect sites within the body. The data presented last year showed this system takes advantage of a radical-mediated chemical reaction that selectively bonds thiols to molecules containing carbon-carbon double bonds ("enes"). This "thiol-ene" reaction can be initiated with exposure to biologically compatible wavelengths of UV light (~365nm, at $10mW/cm^2$) to form PEG hydrogel matrices. Gelation of these synthetic 3D matrices can be accomplished in the presence of cells within minutes making it a suitable vehicle for cell delivery. Moreover, this chemistry leads to the formation of hydrogel niches whose structures can be tuned to promote chondrocyte survival and proliferation on a timescale commensurate with extracellular matrix (ECM) production for cartilage regeneration.

Additionally, growth factors can be tethered into the hydrogel system to provide a local and sustained presentation to encapsulated chondrocytes. Porcine chondrocytes encapsulated in non-degradable thiol-ene gels with tethered TGF-β1, exhibited increased proliferation (Figure 4) as well as enhanced matrix deposition (Figure 5) over a 28 day period compared to systems without the growth factor. Furthermore, markers implicated that the cells maintained an articular cartilage phenotype in the system (Figure 6).

A non-degradable system yielded enhanced matrix production with encapsulated chondrocytes; however, literature suggests cells should generate more ECM in a locally, enzymatically degradable system. A tunable aspect of these hydrogels is the rate of enzymatic cleavage of the crosslinking peptides, which can be tailored by altering the amino acid sequence of the site. In our work with the porcine chondrocytes, we have selected the previously used non-specific matrix metalloproteinase (MMP) sensitive sequence, GPQGIAGQ but this sequence can be easily altered to either increase its susceptibility to enzymatic cleavage and matrix degradation or to slow these processes. Another tunable aspect of this synthetic hydrogel system is the matrix density and stiffness. By using PEG monomers with different arm numbers and concentrations, it is possible to tune global physical properties of the resultant polymers. These data will be used to identify ideal formulations for chondrocyte delivery, with the expectation that targeting the physical properties of the materials to closely match the in vivo environment will provide the best reparative effects.

Subtask 1.1.c Evaluate cartilage and bone matrix produced in vivo in mice.

- Cells encapsulated in photochemically crosslinked gels implanted in mice for matrix production in vivo.

Results: The previous design of photochemically crosslinked collagen, dual, and fibrin gels yielded inconsistent results in both mice and swine. The model was reevaluated in order to present stable gels for objective in vivo studies. The model was changed from the rings of devitalized native articular cartilage rings to 300μL cubes of the gel only, and the gels were immediately implanted into nude mice as opposed to the in vitro culture period. The pilot study was a short 4 week evaluation of the gels in vivo. Swine chondrocytes were encapsulated in 4 percent bovine collagen gels, bovine fibrin gels, and human fibrin gels that were photochemically crosslinked with riboflavin and blue light in hypoxic conditions. Control gels were not photochemically crosslinked. New cartilage matrix was formed in vivo in mice after 4 weeks (Figure 7). New fragmented cartilage matrix was observed in bovine fibrin and collagen gels, both crosslinked and non crosslinked. The crosslinked human fibrin gels could not be harvested after 4 weeks. This work was repeated as a full study. The same model was used (300μL cubes, immediate implantation) and the bovine fibrin and collagen gels were repeated for 6 and 12 weeks in vivo. New fragmented cartilage formation was formed in vivo in mice after 6 and 12 weeks in the fibrin gels, both crosslinked and non crosslinked (Figure 8 and 9). As seen in the gross images, the fibrin gels retained their shape better than the collagen gels. The biochemical and biomechanical analyses of the full study is currently underway.

Subtask 1.2.a Initiate 3-month pilot study in swine with photochemically crosslinked collagen and PEG gels with bilayer of chondrocytes and osetoblasts

- Cells were encapsulated in photochemically crosslinked gels and implanted in swine to evaluate the survival of the cells in vivo.

Result: We reported on the development of the swine model in year 1 showing that four defects could be made in the patella (trochlear) groove and two defects could be made in the medial condyle (Figure 10; left). We also showed in a pilot study that matrix was evidenced in the specimen implanted in photochemically crosslinked riboflavin gel at 4 weeks (Figure 10; middle and right). In year 2, we made modifications to the gels because the photochemically crosslinked collagen gel was too weak to remain in the defect, and performed a pilot study in 2 swine to evaluate cell survival after implantation of the cell-scaffolds. Those gels were carefully removed from the defects and evaluated with a live-dead assay

to assess the survivability of the implanted cells. That short-term pilot study (reported in year 2) confirmed that the cells survive the gelation and implantation process. However, the size (6.5 mm diameter) of the defects was deemed too large to get any meaningful regeneration of the articular cartilage surface. Since we are unable to limit weight bearing (as would be done in humans) in the swine, it is possible that the failure to regenerate full may be related to the significant mechanical forces on the defects in the joint. In consultation with our clinical collaborators, we decided to decrease the size of the defects to 1-2 mm in two swine done in this fiscal year of the grant. This approach was suggested in the conclusions to last year's report. Instead of 6 large defects in the trochlear groove and the condyle of the femur, we made nine, small 1 mm defects in the cartilage surface. These were either left untreated or treated with collagen gel containing cells (Figure 11). Untreated defects showed clefts in the cartilage surface at the time of harvest confirming that no treatment resulted in lesions that do not heal. Those defects treated with the collagen gel containing cells showed early cartilage formation in the defects, and the matrix stained positive for GAG production as demonstrated with Safranin O staining (Figure 11).

Task 2 Stimulation of chondrogenesis by stem cells in photochemical gels

Subtask 2.1.a Perform initial study of collagen and PEG gels with stem cells implanted in mice

- MSCs be harvested from donor swine and grown in culture. Cells encapsulated in gels and implanted in mice for matrix production in vivo.

Results: BM-MSCs were isolated from swine bone marrow collected by aspiration from the iliac crest of swine. The MSC population was isolated by attachment to plastic culture dishes and propagated in high glucose DMEM. Results presented in year 2 demonstrated the multilineage potential of swine BM-MSCs, the cultured cells were placed into commercial differentiation media from Invitrogen to differentiate them into osteogenic, adipogenic and chondrogenic lineages. The results demonstrate that the isolated swine BM-MSCs were differentiated into the osteogenic lineage demonstrated by the increased production of alkaline phosphatase; into the adipogenic lineage with the formation intracellular lipid; and the chondrogenic lineage as demonstrated by the production of cartilage-specific extracellular matrix. In year 3, MSCs were placed into 3D alginate gels and stimulated with chondrogenic media from Invitrogen. After 2 weeks of culture, the gels were placed in vivo into nude mice for 5 weeks. Control gels were made with swine chondrocytes (positive control) and non-differentiated MSCs (negative control). The results are shown in figure 12 showing that gels containing MSCs stimulated with chondrogenic factors produced new cartilage matrix, whereas MSCs in the gels that were not treated with chondrogenic factors failed to make cartilage matrix.

KEY RESEARCH ACCOMPLISHMENTS

- Isolation of MSCs and stimulation towards osteogenesis in vitro on an octacalcium phosphate scaffold
  - Cells populated the scaffold as shown with live-dead assays.
  - Calcium deposits demonstrated with von Kossa stains

- A degradable form of photochemically crosslinked PEG norbornene gel was formulated and growth factors tethered to the polymer.
  - Pericellular cartilage matrix was demonstrated around the encapsulated swine chondrocytes
  - Tethered TGF beta persisted longer than soluble growth factor

- Formation of new cartilage matrix was demonstrated in vivo in mice using photochemically crosslinked gels and swine articular chondrocytes

- Chondrocytes encapsulated in photochemically crosslinked hydrogels can survive the crosslinking and implantation process as shown in swine
  - The size of the defects has been reduced from 6.5 mm down to 1-2 mm in diameter

- Bone marrow-derived mesenchymal stem cells from swine were encapsulated in alginate, stimulated with chondrogenic growth factors, and placed in vivo in mice for new matrix formation

REPORTABLE OUTCOMES

Abstract presented (poster) (Appendix A):
Photochemical Crosslinking Stabilizes Protein Hydrogels for Articular Cartilage Regeneration"
Omobono MA, Jang S, Randolph MA, Zhao X, Redmond RW, Gill TJ
Orthopedic Research Society, San Antonio, TX, February 2013

Abstract presented (Appendix B):
Photochemical Crosslinking Stabilizes Protein Hydrogels for Articular Cartilage Regeneration"
Zhao X, Omobono MA, Jang S, Randolph MA, Redmond RW, Gill TJ
Plastic Surgery Research Council, Santa Monica, CA, May 2013

Manuscript submitted to Journal of Biomedical Materials Research (Appendix C):
Covalently tethered TGF-β1 with encapsulated chondrocytes in a PEG hydrogel system enhancesextracellular matrix production
Sridhar BV, Doyle NR, Randolph MA, Anseth KS

Manuscript in review Biomaterials (appended to year 2 report):
Enhancing the stiffness of collagen hydrogels for delivery of encapsulated chondrocytes to articular lesions for cartilage regeneration.
Omobono MA, Zhao X, Furlong MA, Kwon CH, Randolph MA, Gill TJ, Redmond RW,

CONCLUSIONS

At the conclusion of the third year of this grant award, we made modifications to the formulations of photochemically crosslinked hydrogels that will be used to deliver chondrocytes to articular cartilage defects in the swine knee joint. Preliminary data from year 2 showed that performing the photochemical crosslinking in an oxygen free environment improved cell survival in collagen gels, fibrin gels, and combination collagen-fibrin gels. This was accomplished by flushing the ambient air out of a chamber in which the gels were placed and replacing the gas with 100% nitrogen gas during the irradiation process. Results from year 3 showed that new cartilage matrix was formed in vivo in mice using this new polymerization protocol. As we described in our testing paradigm shown in figure 1 of this report, gels that do not perform well in vitro or in vivo in mice will be reformulated and tested before moving into large animal swine studies. With the modifications described in this report, we will complete large animal testing in year 4.

A photopolymerizable PEG thiol-ene hydrogel was utilized as a platform to covalently attach a growth factor to provide a local and sustained presentation to chondrocytes for cartilage tissue engineering applications. Porcine chondrocytes were encapsulated in a system with immobilized transforming growth factor beta 1(TGF-β1) to enhance proliferation and matrix production. Chondrocytes were encapsulated in hydrogels with and without tethered TGF-β1 with some groups being exposed to solubly delivered growth factor. Over a 28 day period, tethered TGF-β1 promoted proliferation and enhanced matrix deposition with greater efficacy than soluble delivery. These data indicate the potential of PEG thiol-enehydrogels to present TGF-β1 in a manner that locally influences embedded chondrocytes towards a strategy to create bioactive cell delivery vehicles for cartilage tissue engineering.

The original research plan involved making several 6.5 mm osteochondral defects in the trochlear groove and the condyles of the distal femur. We found, however, that the sizes of these defects were too large to permit regeneration of the cartilage surface using any of the gels that we have developed. A pilot study in year 3 reduced the size of the lesions to 1-2 mm with better evidence of regeneration.

The goal of task 2 is to isolate bone marrow derived mesenchymal stem cells from swine and encapsulate these cells in the photochemical gels. In year 2 we showed isolation of swine BM-MSCs that were propagated in culture and differentiated into osteogenic, adipogenic, and chondrogenic lineages using commercial differentiation media containing growth factors. Studies in year 3 report on encapsulating BM-MSCs in the hydrogels and testing these differentiated cells in gels in vivo in mice. Studies in year 4 will focus on evaluating the extracellular matrix formed in vivo by MSCs and performing initial studies of implantation into defects in swine knee joints.

"So What" Section:

In the first and second years of this project our team has developed photocrosslinkable hydrogels that could be used to regenerate cartilage in defects in the articular cartilage surface of joints with the goal of restoring normal joint function. These crosslinkable hydrogels serve as biomimetic polymers that provide a favorable environment for encapsulating chondrocytes (the native cell type found in cartilage) and chondrocyte precursor cells (mesenchymal stem cells). We have successfully formulated collagen and PEG gels, and now a combination collagen+fibrin gel. Whereas we had intended to complete a 3-month study in swine in year 3, the mechanical and biological characteristics were inferior and not suitable for implantation in large animals. According to our iterative testing protocol, work in years 2 and 3 focused on improving the mechanical and biological characteristics of these gels. A pilot study in swine has demonstrated proof of principle that this technology permits cell survival during the photochemical crosslinking process and implantation in swine in the short term (i.e.,

up to 2 weeks). Nonetheless, the size of the cartilage defect proposed in the original application appears to be too large to permit successful regeneration of the joint surface. To resolve this obstacle, we have reduced the size of the defect to 1-2 mm in diameter. We anticipate that this new modification along with changes made in gel formulation and polymerization will be useful adjuncts for joint surface repair and regeneration.

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APPENDIX A

"Photochemical Crosslinking Stabilizes Protein Hydrogels for Articular Cartilage Regeneration"

Omobono, Mark A.[1]; Jang, Seokyoung[2]; Zhao, Xing[1]; Randolph, Mark A.[1]; Redmond, Robert W.[2]; Gill, Thomas J.[1]
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CURRENT PRIMARY CATEGORY: Cartilage & Synovium - Tissue Engineering and Repair
KEYWORDS: Articular Cartilage Repair, Cartilage & Matrix Proteins, Chondrocytes, Tissue Engineering, Knee.

INTRODUCTION: Natural proteins are attractive scaffold materials for tissue engineering purposes. Both fibrin and collagen hydrogels have been investigated as scaffolds for encapsulation of chondrocytes to repair articular surface defects by neocartilage generation. Certain criteria must be met for hydrogels to be suitable for this purpose. The hydrogel must support cell viability and chondrogenesis. It should also retain its shape and structure and resist rapid degradation on implantation and it should possess sufficient mechanical stability for practical handling. Spontaneous fibrin and collagen hydrogels lack one or more of these characteristics. We have found that photochemical crosslinking of biological scaffolds such as fibrin and collagen using visible light and riboflavin-5-phosphate (RF5P) photosensitizer adds some mechanical strength as well as, more importantly, a much greater resistance to enzymatic degradation, allowing the shape and size of the implant to be retained in vitro and vivo. By measuring bulk storage modulus by rheometer, time of degradation using enzymes, and histological staining of devitalized swine cartilage rings loaded with photocrosslinked protein hydrogel in an *in vivo* nude mouse model, we will explore the capacity of this scaffold paradigm for cartilage generation and a potential use in hyaline cartilage tissue engineering. The aims of this study were to determine both the mechanical and biodegradation properties of type-I collagen and fibrin photocrosslinked hydrogels, and to determine whether encapsulated chondrocytes in these gels would generate neocartilage in an *in vivo* nude mouse subcutaneous model using devitalized swine cartilage rings.

MATERIALS AND METHODS: Type-I bovine collagen, fibrin glue, and a 50:50 mixture of collagen and fibrin were combined with 250μM RF5P to create 4.5mm diameter cylindrical hydrogels. Constructs were either non-photocrosslinked (control) or exposed to 20J/cm^2 of visible blue light. For cell-seeded rheology and *in vivo* experiments, fresh chondrocytes were isolated from the patellar groove and trochlear chondyle of 3-4 month old Yorkshire swine and seeded in hydrogel suspension at a density of 40x10^6 cells/mL. Cell-free gels were subjected to enzymatic degradation using 0.025%w/v type-II collagenase or 0.001%w/v papain enzyme and measured for the time of degradation, t_{deg}, on an incubated rocker. Cell-free and cell-seeded gels were also measured for bulk modulus, G', on a TA Instruments AR-G2 rheometer using a frequency sweep from 1-10 radians/second with constant 2% strain rate and an 1000 μm gap under continuous, room-temperature oscillation. Cell-seeded hydrogels were loaded into devitalized swine hyaline cartilage rings for subcutaneous implantation *in vivo* in a nude mouse for 3 weeks. After harvest these constructs were stained with H&E to explore the morphology of the neocartilage.

RESULTS: Photocrosslinked protein hydrogels showed a trend towards higher t_{deg} values, and composite collagen/fibrin constructs showed a 4-fold increase resistance to collagenase degradation when photocrosslinked (Fig 1). There was no significant difference in G' between photocrosslinked and control groups for single-material constructs, but a 18-fold increase in G' was shown after photocrosslinking in cell-less composite constructs, as well as a 9-fold increase in cell-seeded constructs (Fig 2). Constructs harvested from nude mice after 3 weeks showed varying degrees of neocartilage growth within the scaffold matrix. Photocrosslinked collagen and photocrosslinked composite constructs (Fig 3A,C) showed little integration into the devitalized ring and stained strongly for proteoglycans in pericellular regions. Control collagen and composite constructs (Fig3B,D) showed contiguous staining and good integration of the neocartilage matrix into the devitalized ring.

DISCUSSION: Photocrosslinking of protein hydrogels increases the mechanical properties of the scaffold, increases its resistance to biodegradation, and permits neocartilage formation *in vivo*.

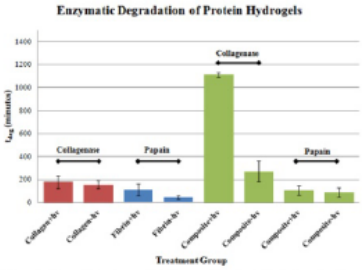

Figure 1: Degradation times of photocrosslinked protein hydrogels of different formulations using type-II collagenase or papain enzyme.

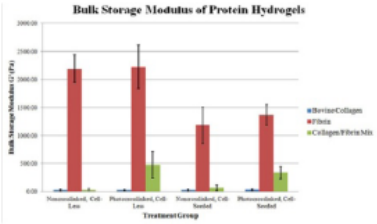

Figure 2: Bulk storage moduli of photocrosslinked protein hydrogels of different formulations using a frequency sweep from 1-10 radians/second with constant 2% strain rate and an 1000 μm gap under

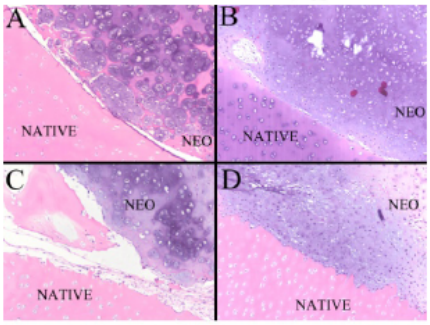

Figure 3: H&E histology of devitalized cartilage rings (NATIVE) loaded with cell-seeded hydrogel scaffold (NEO) after 3 weeks *in vivo*. (A) Photo-crosslinked collagen, (B) control collagen, (C) photocrosslinked composite scaffold, (D) conrol composite scaffold.

APPENDIX B

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PHOTOCHEMICAL CROSSLINKING STABILIZES PROTEIN HYDROGELS FOR CARTILAGE REGENERATION

Presenter: Xing Zhao, MD
Co-Authors: Omobono MA; Jang S; Randolph MA; Redmond RW; Gill TJ; Yaremchuk MJ
Massachusetts General Hospital and Harvard Medical School

Introduction: Photochemical crosslinking of biological scaffolds using visible light and riboflavin-5-phosphate adds mechanical strength as well as, more importantly, a much greater resistance to enzymatic degradation, allowing the shape and size of the hydrogel/chondrocytes implants to be retained both in vitro and vivo, which is a very promising manoeuvre for cartilage regeneration.

Methods: Type-I bovine collagen, fibrin glue, and a 50:50 mixture of collagen and fibrin were combined with 250?M RF5P to create 4.5mm diameter cylindrical hydrogels. Constructs were either non-photocrosslinked (control) or exposed to 20J/cm^2 of visible blue light. Articular chondrocytes were isolated from Yorkshire swine and seeded in hydrogel suspension at a density of 40x10^6 cells/mL. Cell-free gels were subjected to enzymatic degradation using 0.025%w/v type-II collagenase or 0.001%w/v papain enzyme and measured for the time of degradation, tdeg, on an incubated rocker. Cell-free and cell-seeded gels were also measured for bulk modulus, G, on a TA Instruments AR-G2 rheometer using a frequency sweep from 1-10 radians/second with constant 2% strain rate and an 1000 ?m gap under continuous, room-temperature oscillation. Cell-seeded hydrogels were loaded into devitalized swine hyaline cartilage rings for subcutaneous implantation in nude mice for 3 weeks before histological analyse.

Results: Photocrosslinked protein hydrogels showed a trend towards higher tdeg values, and composite collagen/fibrin constructs showed a 4-fold increase resistance to collagenase degradation when photocrosslinked. There was no significant difference in G between photocrosslinked and control groups for single-material constructs, but a 18-fold increase in G was shown after photocrosslinking in cell-less composite constructs, as well as a 9-fold increase in cell-seeded constructs. The in vivo results showed various neocartilage matrix formation and integration status.

Conclusion: Photocrosslinking of hydrogels increases mechanical properties and its resistance to biodegradation, and permits neocartilage formation.

Covalently tethered TGF-β1 with encapsulated chondrocytes in a PEG hydrogel system enhancesextracellular matrix production

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Abstract

A photopolymerizable PEG thiol-ene hydrogel was utilized as a platform to covalently attach a growth factor to provide a local and sustained presentation to chondrocytes for cartilage tissue engineering applications. Porcine chondrocytes were encapsulated in a system with immobilized transforming growth factor beta 1(TGF-β1) to enhance proliferation and matrix production. ELISA results demonstrate that the tethered growth factor was distributed homogenously throughout the gel, and a reporter cell line confirmed that the protein remained bioactive as well as revealeda dose that yielded a maximum cellular response. Chondrocytes were encapsulated in hydrogels with and without tethered TGF-β1 with some groups being exposed to solubly delivered growth factor. Constructs were assayed for cell viability, DNA content, glycosaminoglycan, and collagen content to quantify cartilage matrix production. Additionally, cell-laden gels were assayed for type I and II collagen production by immunofluorescence to assess whether chondrocytes retained an articular cartilage phenotype. Over a 28 day period, tethered TGF-β1 promoted proliferation and enhanced matrix deposition with greater efficacy than soluble delivery. These data indicate the potential of PEG thiol-ene hydrogels to present TGF-β1 in a manner that locally influences embedded chondrocytes towards a strategy to create bioactive cell delivery vehicles for cartilage tissue engineering.

Keywords: cartilage tissue engineering, chondrocytes, protein conjugation, hydrogels, transforming growth factor-β1

1. Introduction

Healing articular cartilage defects remains a significant clinical challenge because of its limited capacity for self-repair and its mechanical properties that are difficult to emulate.[1] Articular cartilage is an avascular tissue with a sparse population of cells surrounded by an extracellular matrix (ECM) that is regulated by numerous growth factors.[2]Therefore, tissue engineering strategies involving chondrocytes and growth factor delivery may help to improve the treatment of articular cartilage lesions.[3,4]

There is growing interest in methods to sequester and present bioactive therapeutic proteins to chondrocytes immobilized in three-dimensional matrices, specifically for application in the area of regenerative medicine.[5]Cytokines are attractive targets for tissue engineering since they can regulate cellular functions, such as proliferation and matrix production, and are often potent at low concentrations.[6]Many of these proteins are commonly introduced as soluble factors in culture media during *in vitro*experiments; however, *in vivo*, growth factors tend tobe sequesteredin the extracellular matrix,allowing local presentation to cells.[5]

A variety of natural and synthetic materials have been examined as potential cell carriers or as therapeutic agents for cartilage repair.[7,8,9] Hydrogel scaffolds appear to be onepromising class of materials, as gels are defined by their ability to retain water, which often better mimics native tissue microenvironments.[10]Furthermore, poly-(ethylene glycol) (PEG) hydrogels have been used to improve micofracturecartilage regeneration outcomes in human trials.[11]

Hydrogel systems permit sequestration of growth factors via covalent tethering, which can provide advantages compared to other forms of protein delivery. In particular, growth factors are typically cross-reactive with multiple cell types and can have short serum half-lives,limitations that often necessitate localized presentation.[12]Since diffusion of lower molecular weight proteins in hydrogels can be quite rapid, some researchers have usedmicroparticlesfor controlled release

presentation of growth factors to encapsulated chondrocytes.[13]While this approach is quite useful, the process can increase the complexity of scaffold preparation and design, as variability can result from differences in protein loading, and release kinetics as well as the size distribution of loaded microparticles. Therefore, strategies to immobilize growth factors in a bioactive, physiologically relevant context area complementary andimportant step towards directing cells to regenerate cartilage tissue.

As one robust method to create protein functionalized materials, we used a thiol-ene chemistry to incorporate thiolated proteins in PEG hydrogels, since PEG systems have been broadly explored for cell delivery applications. [14,15,16,17]Specifically, PEG hydrogels were synthesized through a photoinitiated step growth polymerization,by reactingnorbornene-terminated PEG macromolecules with a dithiolPEG crosslinker.[18]Thisphotopolymerizable system allows for precise spatial and temporal control over polymer formation,as well as facile encapsulation of cells and biologics. The resulting crosslinkedPEG hydrogel has beenemployed toencapsulate numerous primary cells with high survival rates following photoencapsulation.[10,19]

Previously, our group has successfully incorporated thiolatedTGF-β1 in aheterogenous PEG diacrylate system andshown enhanced chondrogenesis of human mesenchymal stem cells.[20] Here, we hypothesized that presentation of TGF-β1to chondrocytes encapsulatedin PEG thiol-enehydrogels would influence their secretory properties and improve the system's application for cartilage regeneration.

In this work TGF-β1 was thiolated and incorporated into a PEG thiol-ene hydrogel. We selected TGF-β1 because it has been shown to increase chondrocyte proliferation and cartilage ECM production in both 3D[13] and 2D studies.[21]We confirmed the presence of tethered TGF-β1 in the

gel by ELISA and investigatedits bioactivity using a PE25 cell reporter assay for SMAD2 signaling.[22]

We demonstrate the clinical potential of the material platform by encapsulating chondrocytes. Specifically, we found chondrocytes encapsulated in gels with tethered TGFβ-1had higher DNA content with higher cell survival throughout all conditions. Additionally, cells cultured in tethered gels produced higher levels ofglycosaminoglycans and collagen compared to gels without TGFβ or TGFβ delivered solubly in the media.The protein functionalizedgels also led to an articular chondrocyte phenotype, as indicated by high levels of type IIcollagen synthesis and minimal levels oftype I collagen.[23]Collectively, these results suggest the potential for tethering growth factor to PEG-based cell delivery vehicles for tunable control of bioactive signals in a local and sustained manner for influencing tissue regeneration.

2. Materials and Methods

PEG monomer synthesis

8-arm polyethylene glycol (PEG) aminenorborneneM$_n$10,000 was synthesized as previously described.[16] Briefly, 5-norbornene-2-carboxylic acid(predominantly endo isomer, Sigma Aldrich) was first converted to a dinorbornene anhydride using N,N'-dicyclohexylcarbodiimide (0.5 eq. to norbornene, Sigma Aldrich) in dichloromethane. The 8-arm PEG monomer (JenKemTechnology USA) was then reacted overnight with the norbornene anhydride (5 eq. to PEG hydroxyls) in dichloromethane. Pyridine (5 eq. to PEG hydroxyls) and 4-dimethylamino pyridine (0.05 eq. to PEG hydroxyls) were also included. The reaction was conducted at room temperature under argon. End group functionalization was verified by ^{1}H NMR to be >90%. The photoinitiator lithium phenyl-2,4,6-trimethylbenzoylphosphinate(LAP) was synthesized as described.[18] The 3.5 kDa PEG dithiol linker was purchased from JenKem Technology.

Cell harvest and expansion

Primary chondrocytes were isolated from articular cartilage of the femoral-patellar groove of 6 month old Yorkshire swine as detailed previously.[24] Cells were grown in a culture flaskin mediaas previously described.[25] Briefly, cells were grown in DMEM growth medium (phenol red, high glucose DMEM supplemented with ITS+Premix 1%v/v(BD Biosciences), 50μg/mL L-ascorbic acid 2-phosphate, 40 μg/mL L-proline, 0.1μM dexamethasone, 110μg/mL pyruvate, and 1% penicillin-streptomycin-fungizone with the addition of 10ng/mL IGF-1 (Peprotech) to maintain cells in dedifferentiated state. ITS promotes formation of hyaline cartilage over serum.[26] Cultures were maintained at 5% CO_2 and 37°C.

Mink lung epithelial PE25 cells containing a stably transfected luciferase reporter gene for TGF-β1were cultured in low glucose DMEM supplemented with 10% fetal bovine serum, and 1% penicillin-streptomycin-fungizone. Cells that were passaged three times were used in encapsulation experiments.

PEG hydrogel synthesis and growth factor incorporation

2-Iminothiolane (Pierce) was used to thiolatehuman TGF-β1 (Peprotech). Briefly, 2-Iminothiolane was reacted at a 4:1 molar ratio to TGFβ for 1 hour at RT. Thiolated TGFβ was pre-reacted at various concentrations with PEG norbornene monomer solution prior to cross-linking via photoinitiatedpolymerization with UV light (I_o~3.5 mW/cm^2 at λ=365 nm) and 0.05 wt% LAP for 30s. The monomer solution was thencrosslinked with a 3.5 kDa PEG dithiol at a stoichiometric ratio of [40 mMdithiol]: [80 mMNorbornene]in a 10wt% PEG solution using longwave ultraviolet light (I_o~3.5 mW/cm^2 at λ=365 nm) for 30s. (Scheme 1)

Quantifying growth factor incorporation

10 wt% hydrogels were synthesized with tethered TGF-β1 at 0, 10,50, or 90 nMandprepared for cryosectioning as previously described.[27] Briefly, hydrogels were flash frozen in liquid nitrogen and placed in HistoPrep (Fisher Scientific) in cryomolds.20μm cross-sections along the plane of the construct were collected on SuperFrost® Plus Goldslides (Fisher Scientific).

40μL disc-shaped gels (O.D. ~5mm, thickness ~ 2mm) without encapsulated cells and with varying concentrations of tethered growth factor were sectioned. We collected 20 μm sections from the top, middle, and bottom of gel. To quantify the TGFβ concentration in each section, a modified ELISA was used as previously described.[14] Briefly, sections were blocked for 1 hour at RT in 5% bovine serum albumin (BSA). Sections were washed 3x in ELISA buffer (0.01% BSA, & 0.05% Tween-20 in PBS) prior to incubation with a mouse anti-human TGF-β1 antibody (Peprotech) at 1:100 dilution overnight at 4 ^{0}C. Sections were washed again, then incubated with goat anti mouse–HRP (eBioscience) for 1 hour at RT and washed again. Sections were incubated with 100μL of peroxidase and 3,3',5,5' tetramethylbenzidine substrate until color developed thenstopped the reaction using 100 μL 2N sulfuric acid. The absorbance was measured at 450 nm using a Bio-Tek H1 spectrophotometer.

To calculate the theoretical loading of growth factor in each section, we determined the volume, assuming the section was a thin disc with a 5mm diameter and 20μm height. Using $V = \pi r^2 h$ and the molecular weight of TGF-β1 (M_n=25,000 g/mol), we calculated the amount of growth factor per section in nanograms. For instance, a 50 nM 40 μL gel section is expected to have 0.5 ngof TGF-β1 per 20 μm section assuming ideal conditions.

Finally, a standard curve was made simultaneously by prepping 96 well high binding clear plates with known amounts of TGF-β1. The 0 nM value at 450 nm was subtracted out from all values in the curve.

TGF-β1 bioactivity and cellular signaling

PE25 cells were encapsulated in 10 wt% gels functionalized with the 1 mMCys-Arg-Gly-Asp-Ser (CRGDS) peptide to promote survival, and 0, 12.5, 25,50, or 100 nMthiolated TGF-β1. Additionally, cells encapsulated in PEG gels without tethered growth factor were exposed to soluble TGF-β1 at concentrations of 0, 0.2, 0.3, 1, or 2 nM. We photo-encapsulated cells at a density of40 million cells/mL, and cell-laden hydrogels were formed in syringe tips at a volume of 40μL. Following encapsulation, hydrogels were placed into DMEM growth medium in 48-well plates and incubated overnight at 37°C, 5% CO_2. Afterwards, hydrogels were incubated in Glo-Lysis buffer (Promega) for 10 min at 37°C; the samples were centrifuged for 10 min (13,400 rpm.4°C); the lysate was transferred to white 96 well plate (50 μL per well), and 50 μL luciferase substrate (Promega) was added to the lysate for 5 min and luminescence was quantified between 300-700 nm.

Chondrocyte encapsulation in PEG thiol-ene hydrogels

Chondrocytes were encapsulated at 40 millioncells/mL in 10 wt% monomer solution and thiolatedTGF-β1 at concentrations of 0 or 50 nM. 40μL cell-laden gels were immediately placed in 1mL DMEM growth medium (without phenol red) in 48 well non-treated tissue culture plates. As a positive control, a subset group of gels without tethered growth factor was exposed to 0.3 nM(7.5 ng/mL) soluble TGF-β1.Media was changed every 3 days. Samples were collected at days 1, 14, and 28 for analysis of ECM production and chondrocyte proliferation. At day 1 and 28 cell viability was assessed using a LIVE/DEAD® membrane integrity assay and confocal microscopy.

Biochemical analysis of cell-hydrogel constructs

Cell-laden hydrogels were collected at specified time points and snap frozen in LN_2,and stored at -70°Cuntil analysis. Hydrogels weredigested in enzyme buffer (125 µg/mL papain [Worthington Biochemical], and 10 mM cysteine) and homogenized using 5 mm steel beads in a TissueLyser(Qiagen). Homogenized samples were digested overnight at 60°C.

DNA content was measured using a Picogreen assay (Invitrogen). Cell number was determined by assuming each cell produced 7.7 pg DNA per chondrocyte.[28]Sulfated glycosaminoglycan (GAG) content was assessed using a dimethyl methylene blue assay as previously described.[29]Collagen content in the gels was measured using ahydroxyproline assay, where hydroxyproline is assumed to make up 10% of collagen.[30]All quantities were normalized per gel and per cell.

Histological and immunohistochemical analysis

On day 28, constructs (n=2) were fixed in 10% formalin for 30 min RT, then snap frozen and cryosectioned. Sections were stained for safranin-O or masson'strichrome on a Leica autostainer XL and imaged in bright field (40X objective) on a Nikon inverted microscope.

For immunostaining, sections were blocked with 10% goat serum, then analyzed by anti-collagen type II (1:50, US Biologicals) and anti-collagen type I (1:50). Sections were treated with appropriate enzymes for 1 hour at 37 °C : hyaluronidase (2080 U) for collagen II, and pepsin A (4000 U) with Retrievagen A (BD Biosciences) treatment for collagen I. Sections were probed with AlexaFluor 568-conjugated secondary antibodies and counterstained with DAPI for cell nuclei. All samples were processed at the same time to minimize sample-to-sample variation. Images were collected on a Zeiss LSM710 scanning confocal microscope with a 20X objective using the same settings and post-processing for all images. The background gain was set to negative controls on blank sections that received the same treatment. Positive controls were

performed on porcine hyaline cartilage for collagen type II and porcine meniscus for collagen type I.

Statistical Analyses

Data are shown as mean ± standard deviation. Two way analysis of variance (ANOVA) with post hoc test for pairwise comparisons was used to evaluate the statistical significance of data, and one way ANOVA to assess differences within specific conditions. $p < 0.05$ was considered to be statistically significant.

3. Results

Distribution of thiolated TGF-β1 in PEG hydrogels

To confirm whether TGF-β1was homogenously distributed within the gel after the thiolenetethering process, a modified section ELISA was employed.[14]The results presented in Figure1 confirm TGF-β1 incorporation throughout the gel, and its relatively homogeneous distribution among gel regions, as well asexperimentally measured values that are similar to theoretically loaded levels (0.1 ng for 10nM, 0.5 ng for 50nM, and 0.9 ng for 90 nM).

Bioactivity and concentration of tethered TGF-β1in 3D culture

We investigated the bioactivity of tethered TGF-β1 in 3D culture using a reporter cell line. Briefly, it has been shown that tethered proteins typically maintainhigh levels of bioactivity when conjugated using thiol-ene reactions.[20]We further determined concentrationsof soluble and tethered TGF-β1that yielded a maximal responsein PE25 cells at a seeding density of 40 million cells/mL. In Figure 2a, there was a significant difference in luciferase output of 50 nM gels compared to other conditions. In Figure 2b, 0.3 nM via soluble delivery elicited a maximal cellular response. Interestingly, when we dosed 50 nM of soluble TGF β-1 to encapsulated PE25s at 40 million cells/mL, the average luciferase response was ~ 6,510 arbitrary units (n=4),

which is a 3-fold lower response than for the same concentration of tetheredTGF-β1. Based on these results, we elected to dose soluble TGF-β1 at the magnitude of 0.3 nM. Overall, these results suggest that tethered TGF-β1 is bioactive, andat 40 million cells/mL, the conditions that elicited the highest responsetoTGF-β1 were 0.3 nM (soluble) and 50 nM (tethered).

Proliferation of chondrocytes exposed to TGF-β1

Cell viability for all encapsulation and culture conditions was between 80%-90% assessed by live/dead membrane integrity assay at both days 1, and 28, and the chondrocytes maintained a spherical morphology. In Figure 3a, one notes the rounded shape of the encapsulated cells, as well as a significant increase in number of cells in the 50 nMTGF-β1 tethered gels. To further quantify this proliferation, we harvested samples at day 1, 14, 28 and assayed for DNA content (Figure 3b). There was a statistically significant increase in DNA content over the 28 day period for cells encapsulated in 50 nM TGF-β1 containing gels. Further, there was significantly more DNA in the day 28 50nM condition than either the 0.3nM or 0nM gel condition ($p<0.001$). Combined with the viability results, these data suggest an increase in chondrocyte proliferation in response to tethered growth factor presentation.

Matrix deposition as a function of TGF-β1 presentation and culture time

Gels were assessed for glycosaminoglycan (GAG)and total collagen contentat day 1, 14 and 28. Encapsulated chondrocytes were either exposed to 0nM, 0.3 nMsolubly or 50 nM tethered TGF-β1. Measured quantities were normalized to cell content in the respective hydrogel formulations. In Figure 4a, GAG production per cell on day 28 for the tethered construct was significantly higher than non-treated groups ($p<0.001$). There was also a significant difference at day 28 between constructs that presented tethered TGF-β1 compared to solubly delivered TGF-β1

($p<0.001$), suggesting that the tethered growth factor enhances ECM production over soluble growth factor delivered in the media.

In Figure 4b, total collagen production per cell washighest at day 28 from the construct with tethered TGF-β1. Further, there was a significant difference between the tethered and soluble TGF-β1 conditions ($p<0.001$) at day 28, and both were significantly different from the 0nM group ($p<0.001$), indicating that collagen content is highest in the tethered protein constructs.

Matrix organization

The distribution and deposition of extracellular matrix molecules were examined by histological and immunofluorescence techniques. Masson's trichrome staining (Figure 5 a,c,e) revealed collagen deposition increased in the pericellular space of encapsulated chondrocytes with both tethered and soluble TGF-β1 gels on day 28 compared to 0nM gels. Overall, it appearsthat most of the pericellular collagen deposition occurs in the 50 nM gels at day 28. In a similar fashion, safranin-O (Figure 5 b,d,f) staining revealed that GAG deposition localized in the pericellularregion with increased deposition per cell in the presence of TGF-β1. These results support the data that tethered TGFβ increases ECM secretion.

Immunofluorescence staining revealed that by day 28 there was a scarce amount of collagen I throughout all samples (Figure 6 a,c,e) and that collagen IIwas prevalent in the growth factor treated samples (Figure 6 d,f) compared to the 0nM sample (Figure 6 b). A high collagen II and low collagen I signal is indicative of articular cartilage, and the constructs maintain that phenotype over 28 days of culture.

4. Discussion

Engineering a clinically viable scaffold for chondrocyte delivery and promotion of cartilage regenerationis challenging, partly because of the time required for chondrocytes to generate a

robust matrix. By encapsulating chondrocytes in a PEG thiol-ene system with localized presentation of growth factor, we have shown quantitatively and qualitatively *in vitro* that cells survive, proliferate, and generate cartilage specific ECM molecules at a higher rate than without the growth factor. Tethering growth factors into a synthetic material scaffoldintegrates both the promoting effects of a protein cross-linked gel without gel to gel variability. A cell delivery system with suchproperties can provide certain advantages for clinical applications.

There are many advantages to tethering growth factors into a gel system for tissue engineering purposes. Localized presentation precludes growth factors from activating unnecessary cell targets in an *in vivo* setting. Additionally, it requires a lower amount of growth factor. In the 28 day study, TGF-β1 is dosed in 1mL media every 3 days at 0.3 nM which results in ~ 70 ng of protein delivered to the cell-laden gel. For the same time period and experimental conditions, a 50 nM tethered gel corresponds to ~50 ng of TGF-β1/gel,yet led to higher matrix production and DNA content at day 28. When using an expensive and/or potent growth factor to promote tissue regeneration, a tethered system canpotentially provide a more efficient and effective delivery system for long time periods appropriate for clinical settings.

In these studies, we chose to look specifically at chondrocytes encapsulated at 40 million cells/mL, since this cell density has been previously shown to be an optimal choice for *in vivo* studies with hydrogel delivery systems. [31, 32,33] We used a cellular assay, based on PE25 cells as a reporter system with a luciferase output, to determine that an effectiveconcentration of growth factor to deliver to cells was 50 nM (Figure 2a) for tethered TGF-β1 and 0.3 nM for solubleTGF-β1(Figure 2b).We hypothesize that encapsulated cells may not respond as well to higher concentrations of soluble TGF-β1 than tethered TGF-β1, because PE25s may internalize the factor, and at a high seeding density not as many cells will generate a response. Related studies

with Mv1Lu cells showed that they internalized TGF-β1, so it is reasonable to consider this explanation for the PE25 experiments.[34]

We speculate that for gels presenting 100 nM of tethered TGF-β1, the PE25s encapsulated at 40 million cells/mLshowed less activity compared to 50 nM gels (Figure 2a) because growth factors can have pleiotropic effects that may lead to a negative feedback loop. Additionally, since TGFβ binds to a dimer receptor, which requires two receptor subtypes to join to initiate the signaling cascade, it is possible that the orientation of growth factors around the cell prevents complete binding since both subtype receptors may be occupied by separate ligands when only one is required for signaling activation.[35]

We chose to use human TGF-β1 with porcine chondrocytes because the PE25 system has already been established with human TGF-β1, [22] and porcine chondrocytes will be used in future pre-clinical animal studies. We believe that this is unlikely to affect the outcomes of our studies, since mature TGF-β1 is known to be highly conserved (>99% amino acid sequence identity) throughout mammalian species.[36]

The data presented in this study suggest that the PEG thiol-ene platform with tethered TGFβ can be a potential tissue engineering system for chondrocyte delivery in a bioactive scaffold. Chondrocytes maintain a spherical morphology, similar to native chondrocytes, in the gel over a 28 day period, as shown in Figure 3a, which suggests the cells are less likely to de-differentiate and generate hyaline-like cartilage.[37]Chondrocytes also increase in cell number when cultured in PEG thiol-ene gels as shown in Figure 3b, and especially whenTGF-β1is presented, which is known to induce proliferation.[21]Porcine chondrocyte doubling time in 2D culture is around 6.4 ±0.3days in serum-containing media.[38]We speculate that part of the reason the cells do not double at a similar rate when encapsulated in the PEG gels is that the selected gel formulations

are non-degradable. Thus, the polymer network limits the amount of space available for chondrocytes to grow, and the media did not contain serum. This result was confirmed by a study with rat chondrocytes grown in a non-degradable 3D scaffoldwhich had a longer doubling time (10.04 ± 0.9 days) than cells grown in 2D (2.94 ± 0.3 days). [39]

Extracellular matrix production data revealed that over 28 days, the tethered-protein gel stimulated chondrocytes to produce more GAGs and collagen, as quantified in Figure 4. A study with juvenile and adult chondrocytes encapsulated in degradable gelshad higher GAG and collagen outputs per cell over a 28 day period compared to the ones in this study. [40]We speculate that a degradable gel allows for greater ECM deposition as posited by various groups.[41,42]Additionally, histology and immunofluorescence staining confirmed that matrix was primarily deposited pericellularlyin all conditions, but at a higher level in gels with tetheredTGF-β1. While the secreted matrix is primarily confined to the pericellular region, there are some areas where the ECM molecules, especially GAGs, are more dispersed between cells (Figure 5). These data suggest the need for tethering TGF-β1 to a degradable PEG thiol-ene system to enhance ECM production and elaboration, with the potential to better mimic native hyaline tissue.

Conclusion

We confirmed that thiol-ene reactions allow conjugation of TGF-β1 into PEG gels, while maintaining bioactivity and the ability to signal to encapsulated cells. We showed that tethered TGF-β1 increasedthe proliferation rate and ECM production of chondrocytes over a 28 day period, at levels exceeding thatof cells in gels where TGF-β1 was dosed in the culture medium or those that were untreated. The tethered TGFβ hydrogels utilize a lower total protein dosage while still promotinghigh levels ofproliferation and matrix production of chondrocytes. Furthermore,

chondrocytes maintain a spherical morphology in the thiol-ene PEG gels with high viability and a phenotype that resembles articular cartilage (i.e. highcollagen II and low collagen I levels are expressed). Collectively, these results demonstrate the feasibility of delivering bioactive protein signals in a 3D culture platform to enhance matrix production of chondrocytes which may have further implications for cartilage regeneration scaffolds *in vivo*.

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Disclosure

No benefit of any kind will be received either directly or indirectly by the authors

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Figure Captions

Scheme 1.Pre-polymerization scheme with thiolated TGF-β1. Initially thiolated TGF-β1 is phototethered into the 8 arm 10 kDa PEGnorbornene network, then the 3.5 kDadithiolcrosslinker is added in with chondrocytes to complete the encapsulation process.Growth factor is not drawn to scale. In featured experiments, there is a lower amount of growth factor attached to the monomer end.Chondrocytes seeded at 40 million cells/mL retain a rounded morphology similar to cells in native tissue. Scale bar represents 50 μm.

Figure 1. TGF-β1 is homogenously distributed throughout the PEG hydrogel. Section ELISA of tethered gels without cells show detection of TGFβ at similar levels to theoretical values with graphic on top depicting slice areas. Each section ~ 20μm. Theoretical values indicated by dashed lines (0.1 ng for 10 nM, 0.5 ng for 50 nM, and 0.9 ng for 90 nM gels). 0nM value is subtracted out of all conditions. Results are presented as mean activity ± s.d. (n=1 gel 3 sections/area). Solid lines indicate p values with one way ANOVA analysis to confirm sections of each gel are not statistically different from each other.

Figure 2.Determining TGF-β1 concentration that yields maximal response.(a)PE25s were encapsulatedat 40 million cells/mL with varying concentrations of tethered TGFβ and 50 nMyielded a maximal response. * indicates statistically significant difference between 50 nM and the other concentrations with $p<0.001$. Results are presented as mean activity ± s.d. (n=4). (b) PE25 cells encapsulated at 40 million cells/mLwere transiently exposed to varying concentrations of TGFβ in the media. The 0.3 nM output is statistically different from the concentrations flanking it. ** indicates statistically significant difference from 0.3 nM to 0.2nM and 1 nM outputs with $p<0.01$. Results are presented as mean activity ± s.d. (n=4).

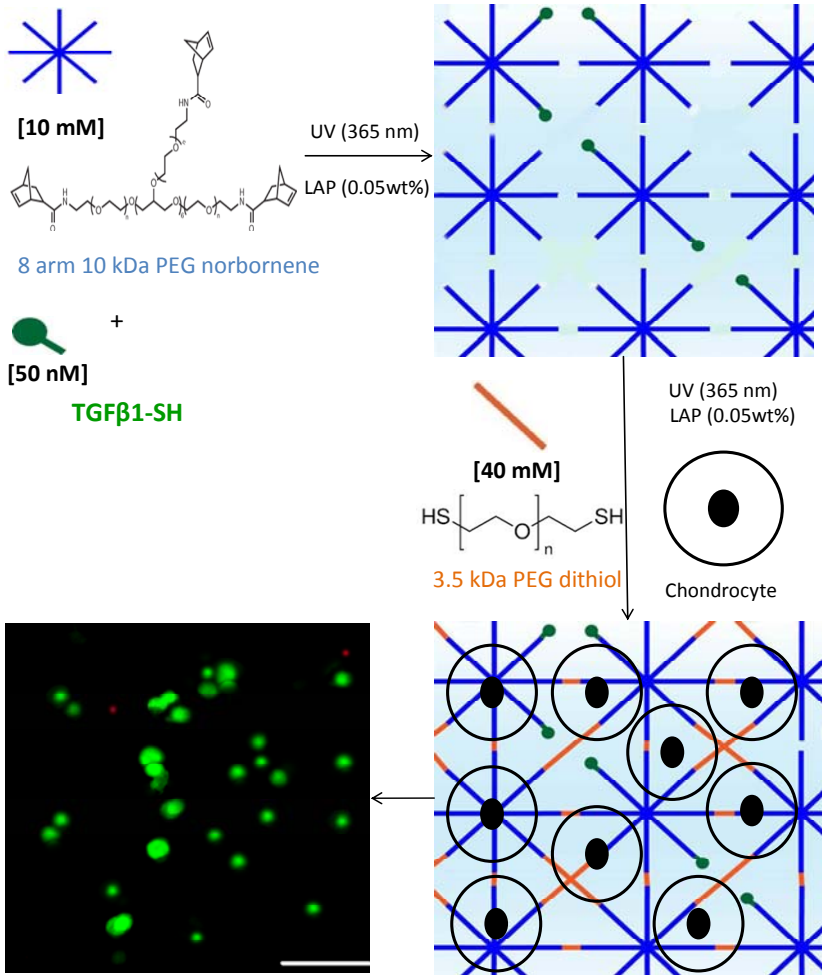
Figure 3.Increased proliferation of chondrocytes exposed to TGF-β1. (a) Live/Dead staining of 50 nM gels seeded at 40 million cells/mL on day 1 and day 28 shows chondrocytes retain a spherical morphology, have high viability, and increase in number. Scale bars represent 50μm. (b) DNA content of chondrocytes encapsulated at 40 million cells/mLthat were exposed to 0nM, 0.3 nM which was delivered through the media, or 50 nM which was tethered into the gel. Over a 28 day period, the cells in the 50 nM condition show a steady rate of increase of DNA content. + indicates significant difference between the 0.3 nM and 0 nM case ($p<0.001$), ++ indicates significant difference between 50 nM and 0nM case ($p<0.001$), * indicates significant difference between 0.3nM and 0nM ($p<0.001$), ** indicates significant difference between 0.3 nM and 50 nM case at day 28 ($p<0.001$), and *** indicates significantdifference between 50 nM and 0 nM for day 28 ($p<0.001$). Results are presented as mean± s.d. (n=7).

Figure 4.Enhanced matrix production of encapsulated chondrocytes exposed to TGFβ. (a) GAG production was normalized per cell. * indicates significant difference between 0.3 nM and 50 nM condition at day 28 ($p<0.001$), ** indicates significant difference between 50 nM and 0nM at day 28 ($p<0.001$). Data presented as mean ± s.d. (n=6). (b) Collagen production was

normalized per cell. + indicates significant difference between 0.3nM and 50 nM at day 28 ($p<0.001$) and ++ indicates significant difference between 0nM and 50 nM at day 28 ($p<0.001$). Data presented as mean ± s.d. (n=5).

Figure 5.Matrix protein distribution ingels. At day 28, gels seeded with chondrocytes at 40 million cells/mL were sectioned and stained for matrix distribution. (a) 0nM gel stained for collagen, (b) 0nM gel stained for GAG, (c) 0.3 nM (soluble) gel stained for collagen, (d) 0.3 nM (soluble) gel stained for GAG, (e) 50 nM(tethered) gel stained for collagen, (f) 50 nM(tethered) gel stained for GAG. Blue indicates collagen and red indicates GAG. Scale bars represent 100 μm.

Figure 6. Collagen I vs. collagen II distribution in constructs. Gels seeded with chondrocytes at 40 million cells/mL were cryosectioned at day 28. Immunohistochemistry analysis reveals collagen type distribution in scaffolds. (a) 0nM with collagen I, (b) 0nM with collagen II, (c) 0.3nM(soluble) with collagen I, (d) 0.3 nM (soluble) with collagen II, (e) 50 nM (tethered) with collagen I, (f) 50nM (tethered) with collagen II. Sections were stained red for both anti-collagen I and anti-collagen II antibodies and were counterstained with DAPI (blue) for cell nuclei. Scale bars represent 50 μm.


[10 mM]
UV (365 nm)
LAP (0.05wt%)
8 arm 10 kDa PEG norbornene
+
[50 nM]
TGFβ1-SH
[40 mM]
HS
O
n
SH
3.5 kDa PEG dithiol
UV (365 nm)
LAP (0.05wt%)
Chondrocyte

Scheme 1

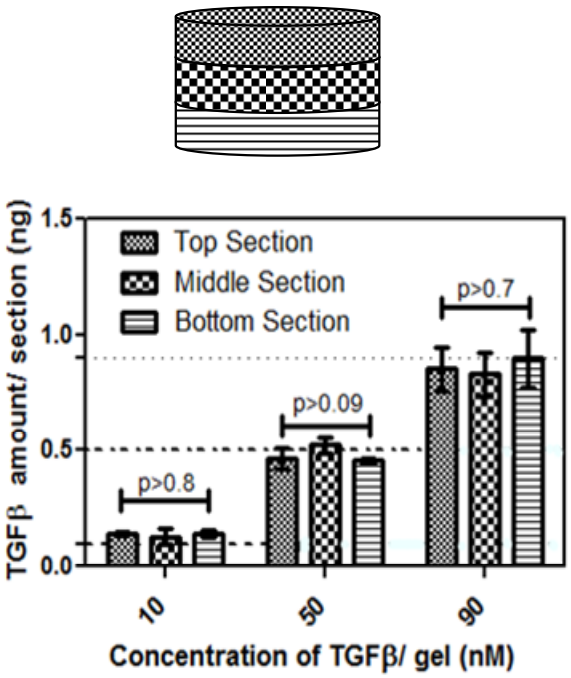
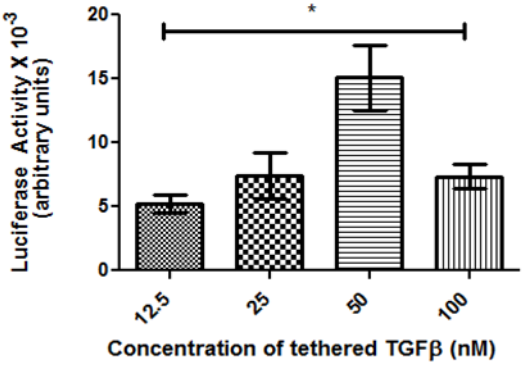


Figure 1

a.



b.

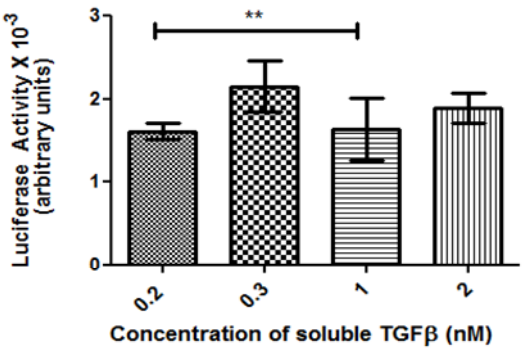
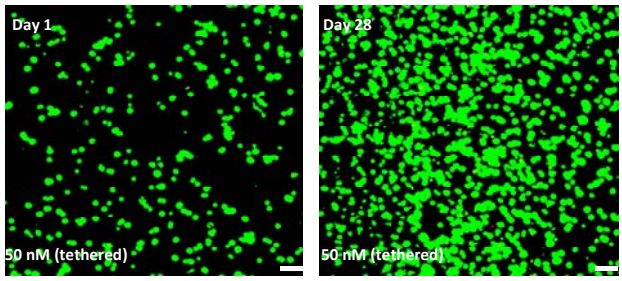


Figure 2

a.



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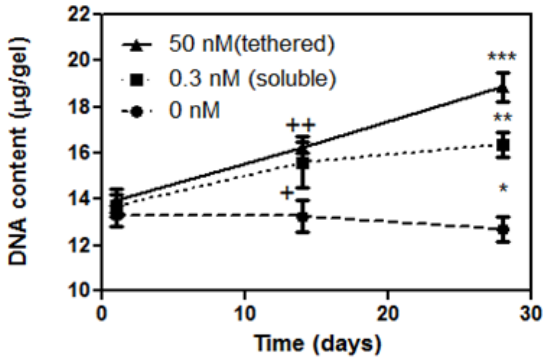
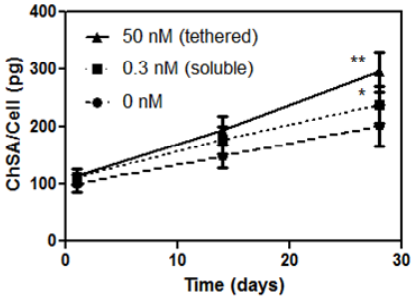


Figure 3

a.



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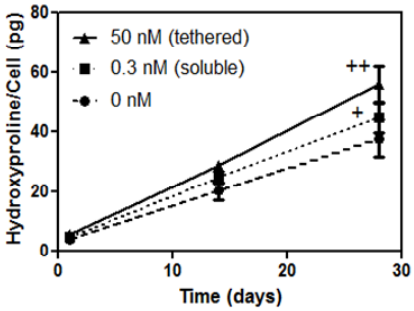


Figure 4

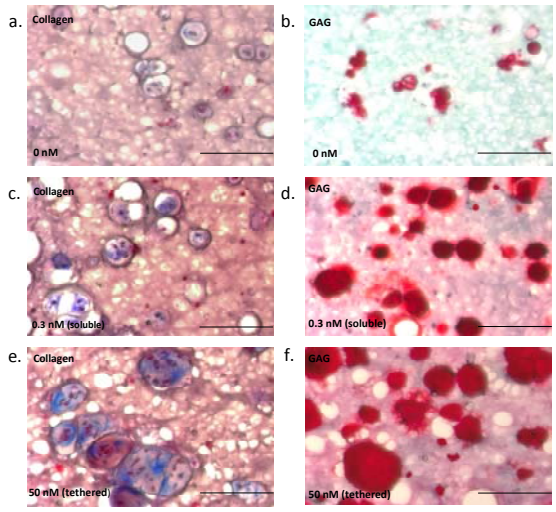


Figure 5

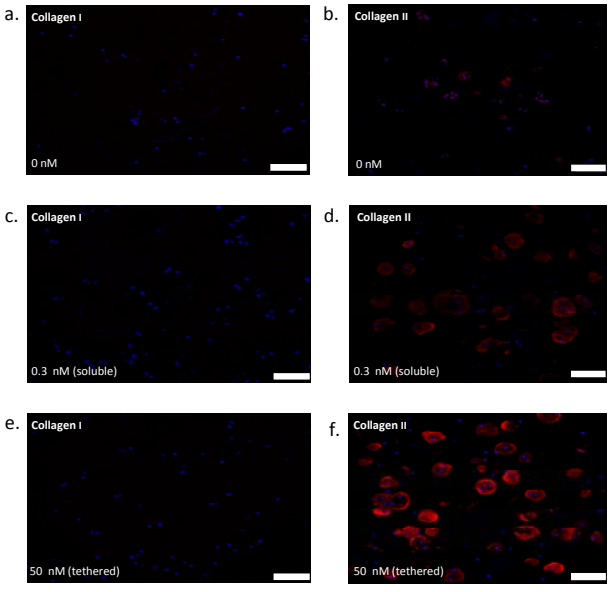


Figure 6

SUPPORTING DATA

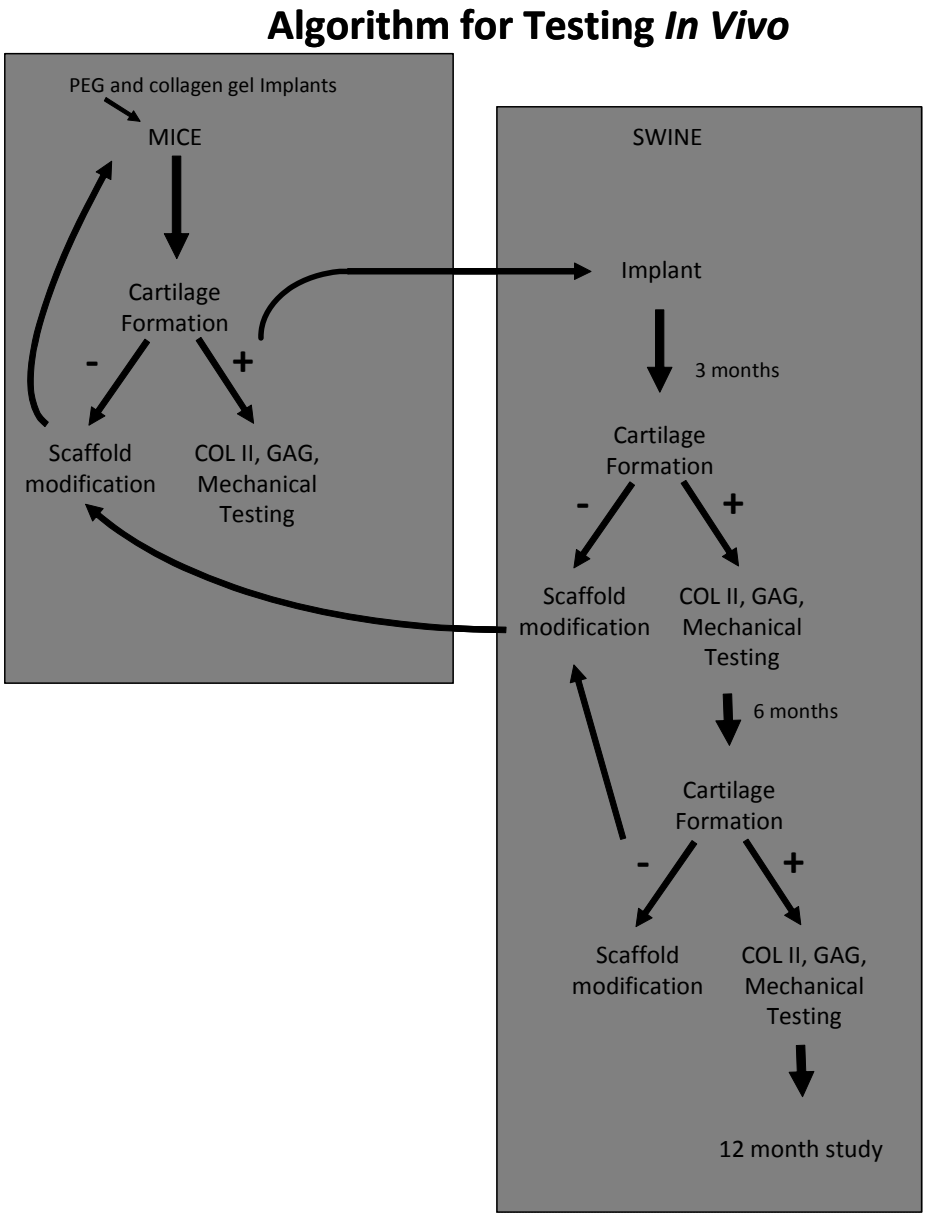
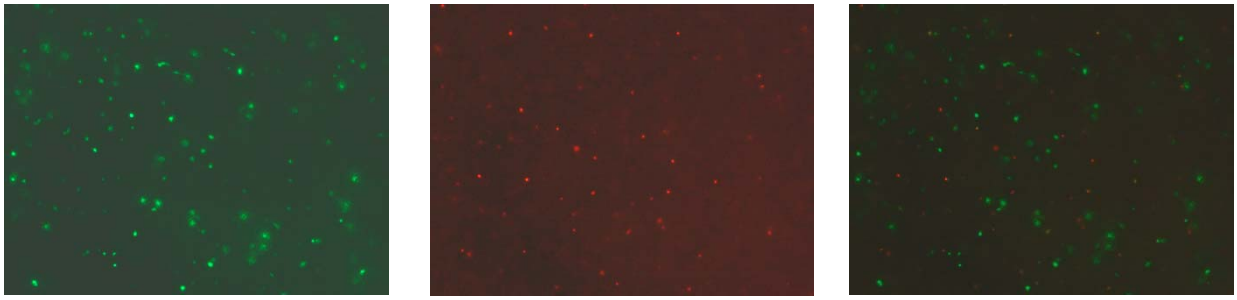


Figure 1. This is the testing paradigm proposed in the original grant application. The gels are made and tested in vitro and in vivo in mice (left box) prior to embarking on the large animal swine model (right box). If gels fail to perform in the mice they are not tested in swine. Additionally, if the gels should perform poorly in the early test phase in swine, the gels are reformulated and tested again in mice before re-embarking on the swine studies. Many changes outlined in this report describe changes to the gels so that large animals are not used unnecessarily for gels that have not been optimized.



Live **Dead** **Merged**

Figure 2. Swine msenchymal stem cells seeded on octacalcium phosphate crystals show high viability immediately after seeding. (All 40x)

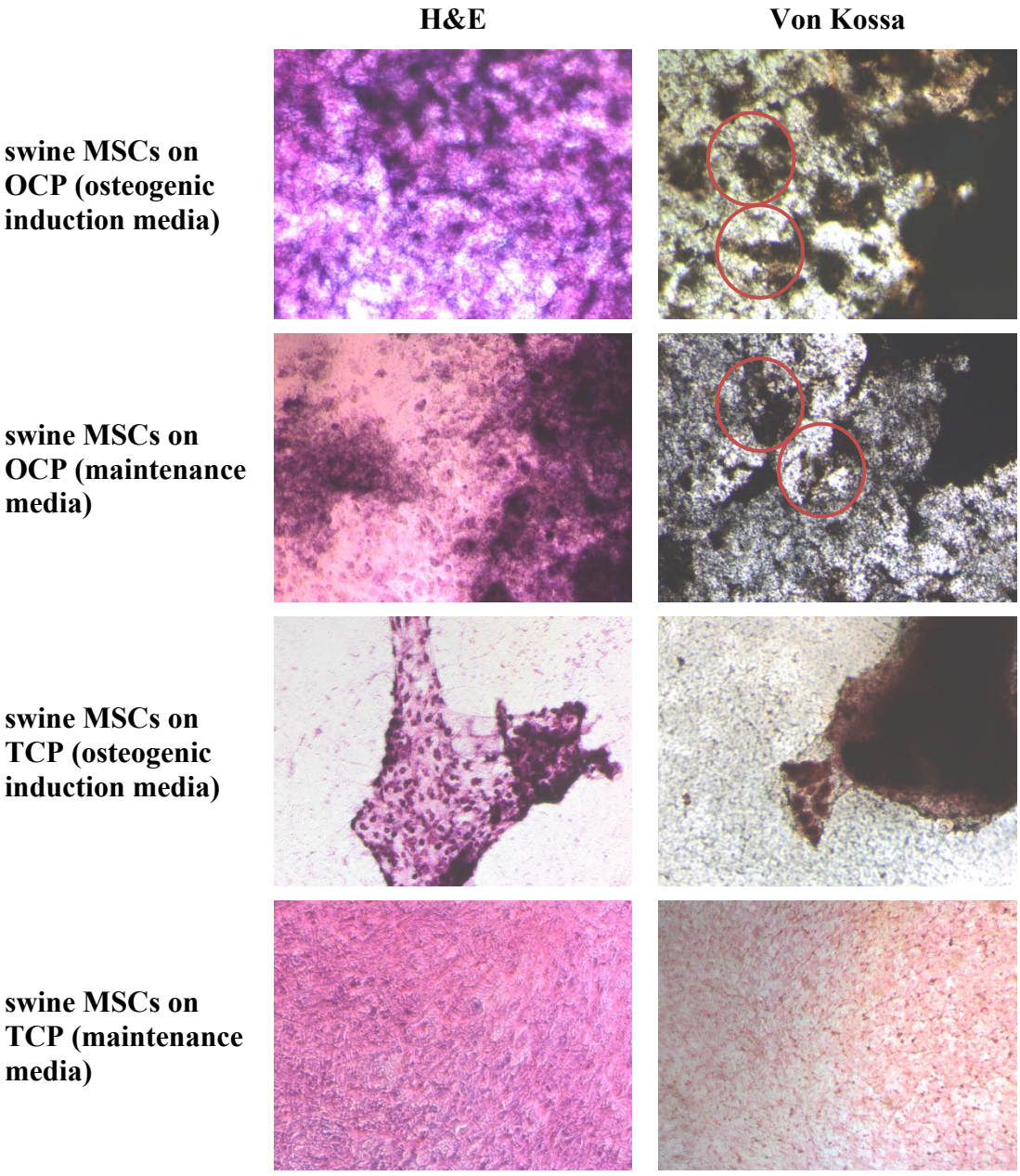
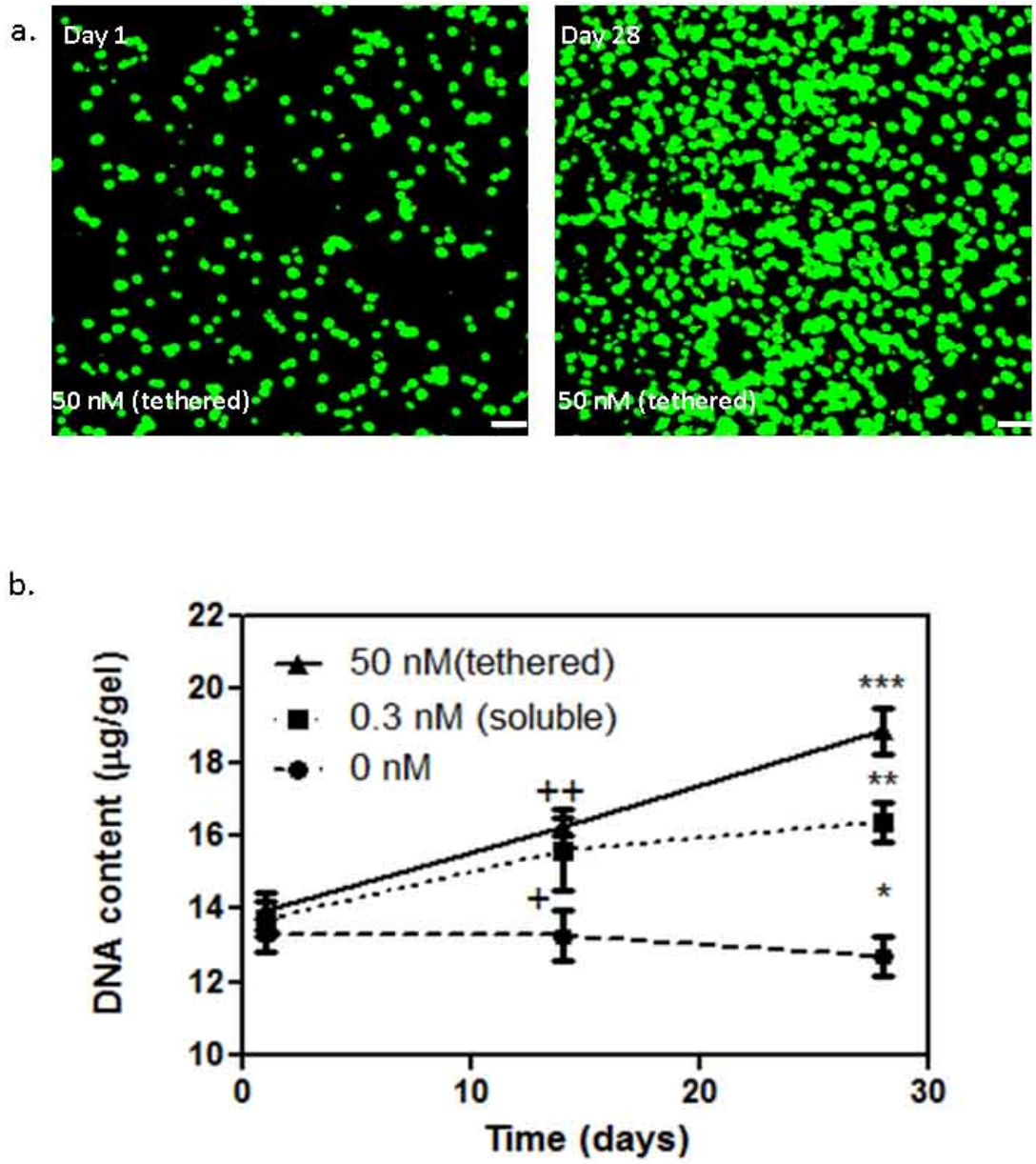


Figure 3. Swine MSCs seeded on octacalcium phosphate crystals (OCP) and tissue culture plastic (TCP) controls. Specimens stained with hematoxylin and eosin and von kossa stains. The von kossa stains for calcium deposits. The red circles are highlighting areas of calcium deposits. (All 100x)



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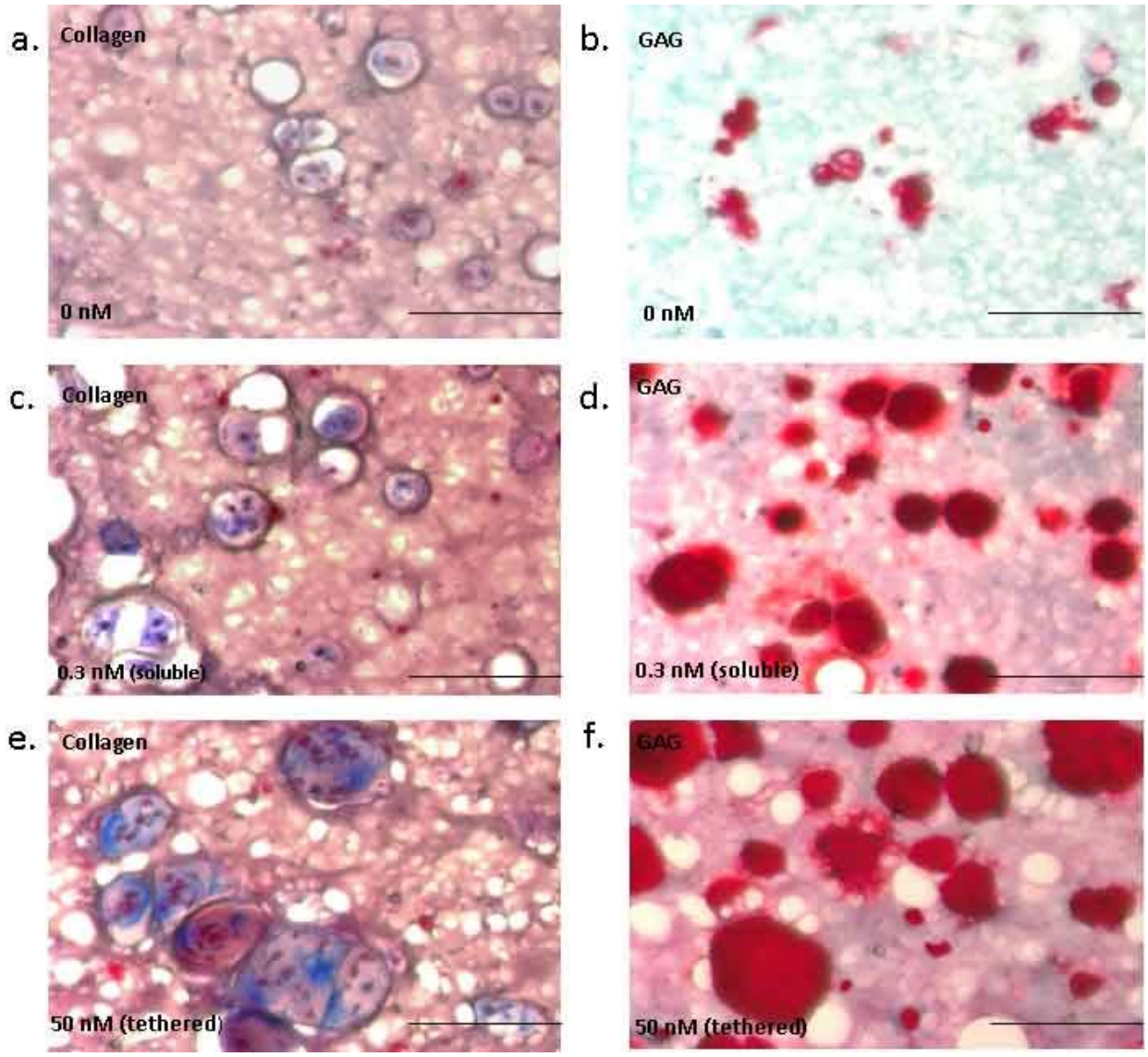

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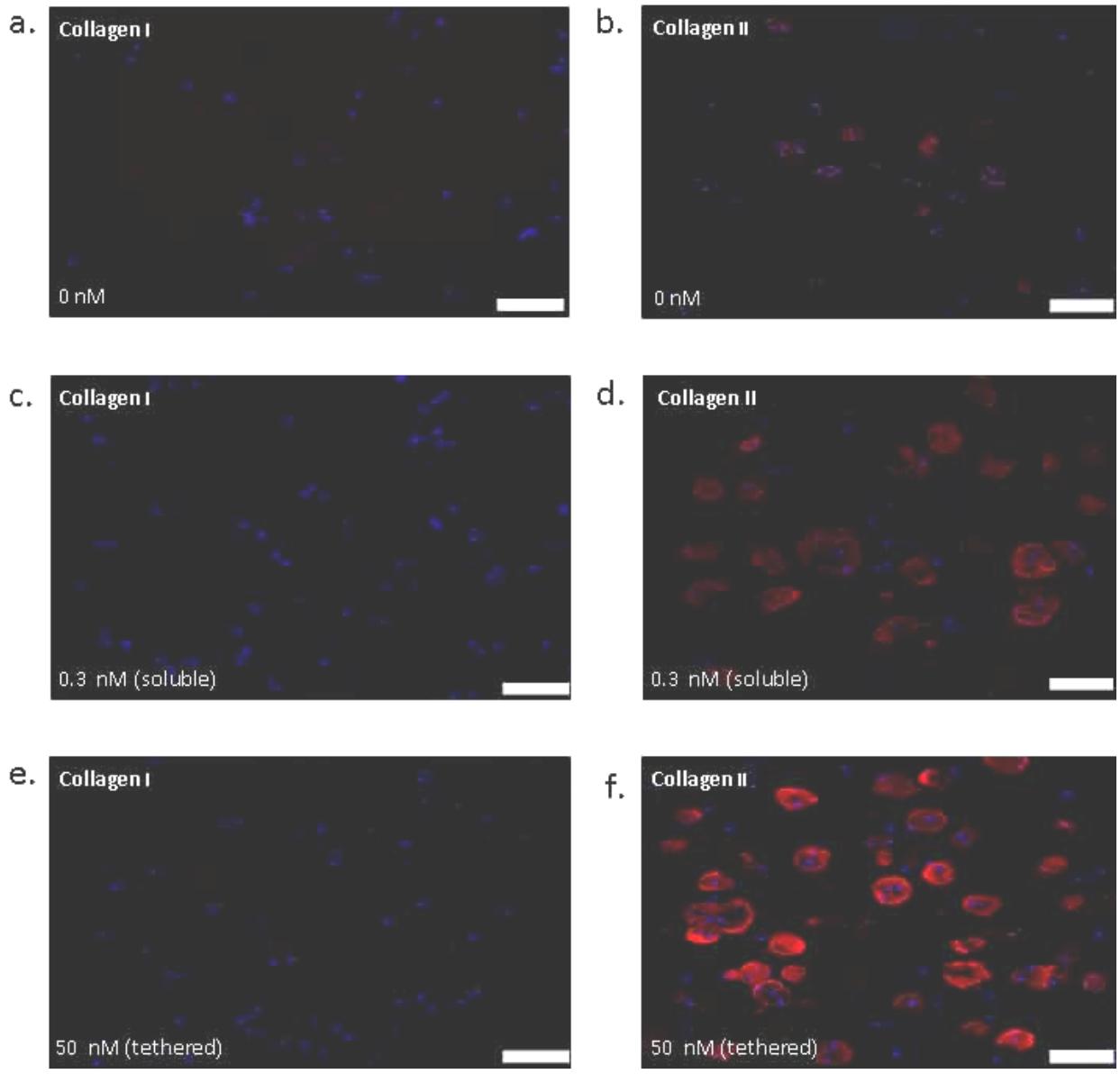

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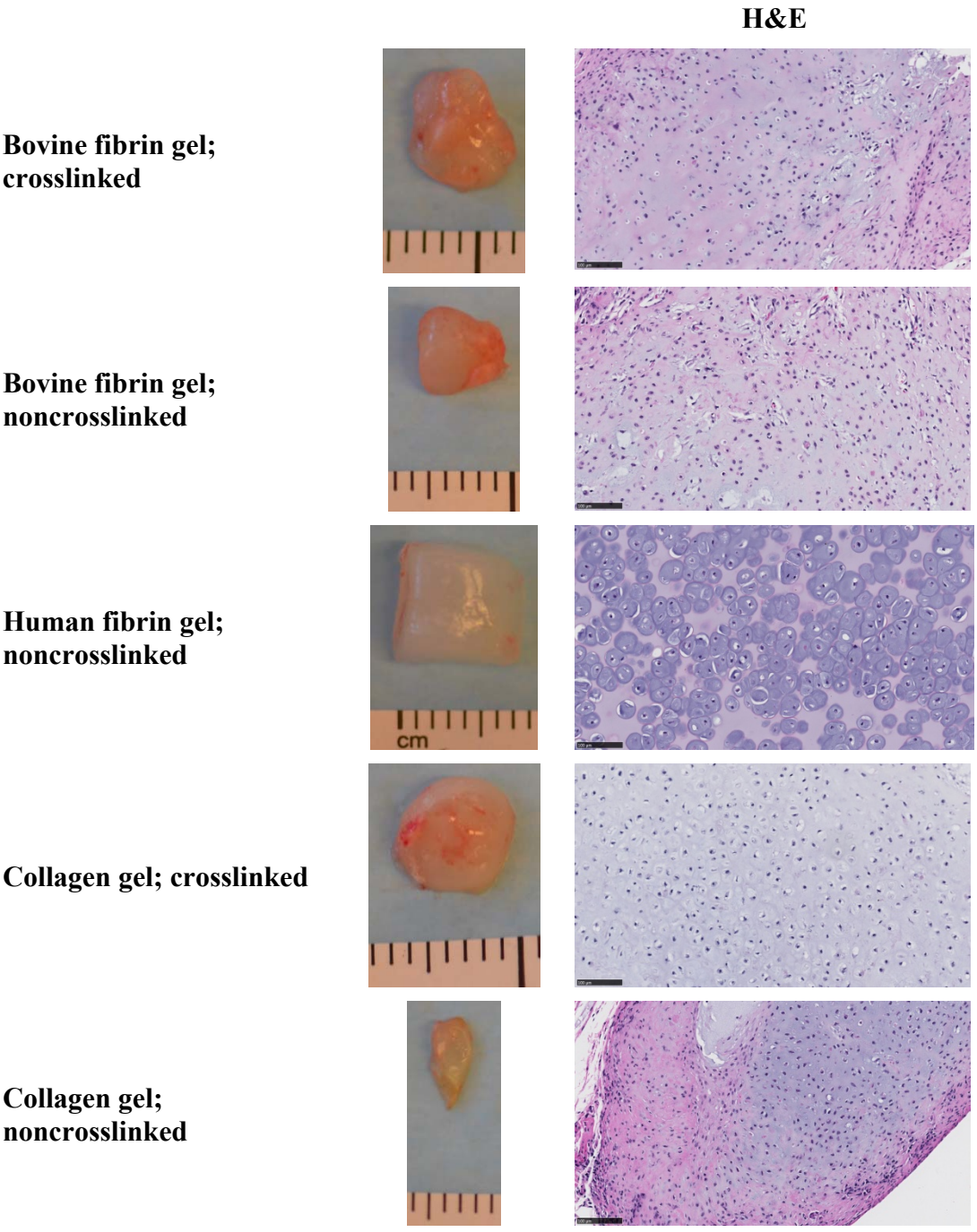


Figure 7. Specimens harvested from mice after 4 weeks in vivo showing the nodule of tissue collected on the left and histological specimens stained with hematoxylin and eosin for each group. (Measure bar is 100 microns.)

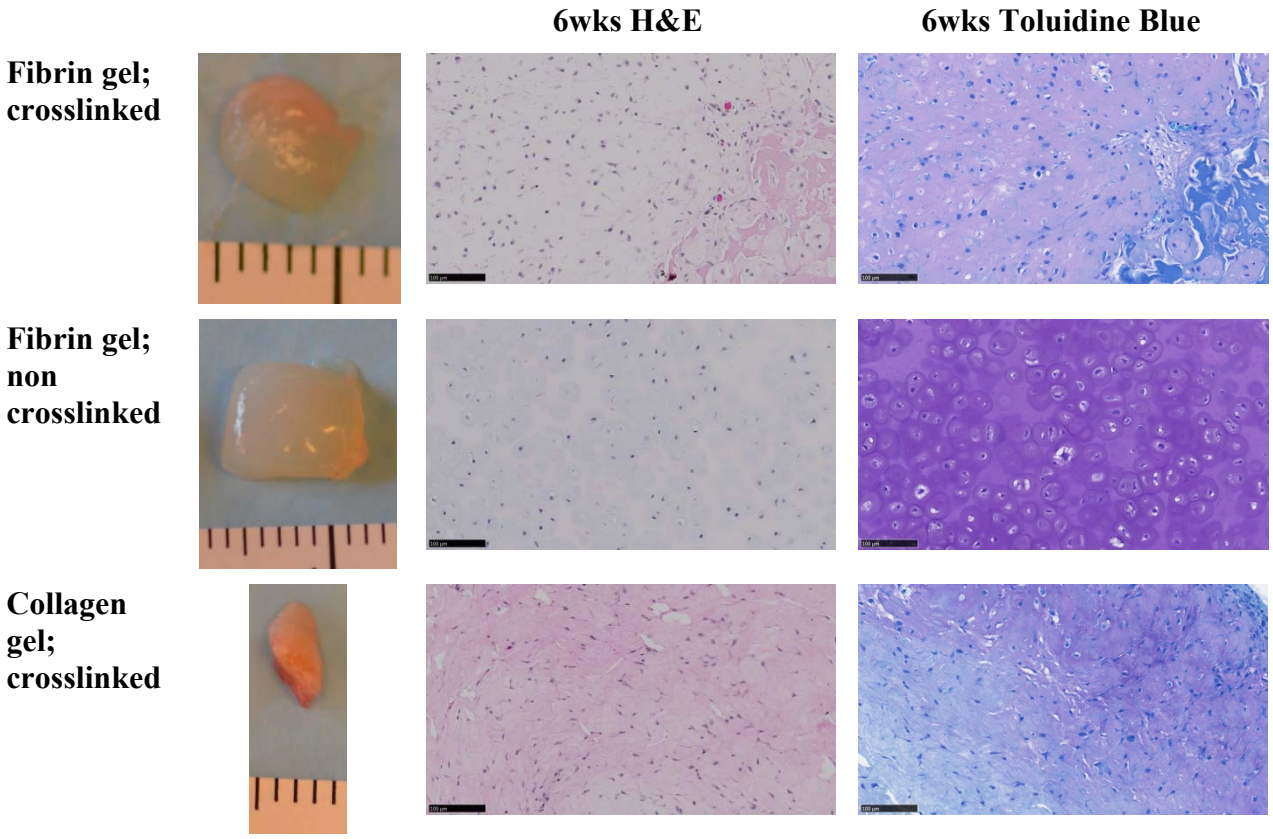


Figure 8. Specimens harvested from mice after 6 weeks in vivo showing the nodule of tissue collected on the left and histological specimens stained with hematoxylin and eosin and toluidine blue for each group. (Measure bar is 100 microns.)

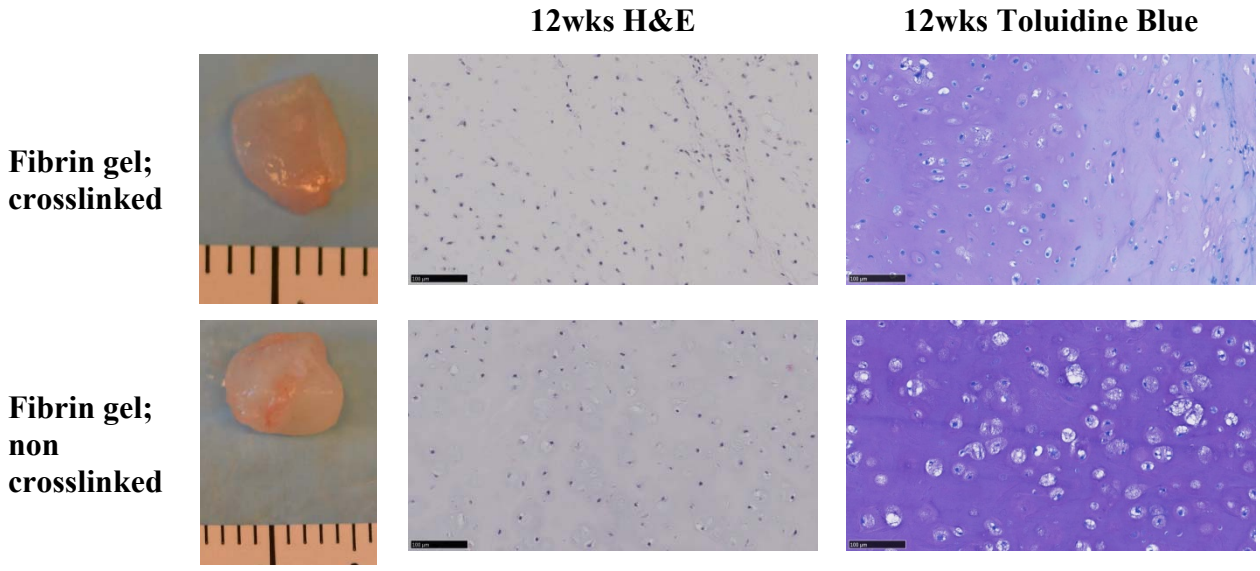


Figure 9. Specimens harvested from mice after 12 weeks in vivo showing the nodule of tissue collected on the left and histological specimens stained with hematoxylin and eosin and toluidine blue for each group. (Measure bar is 100 microns.)

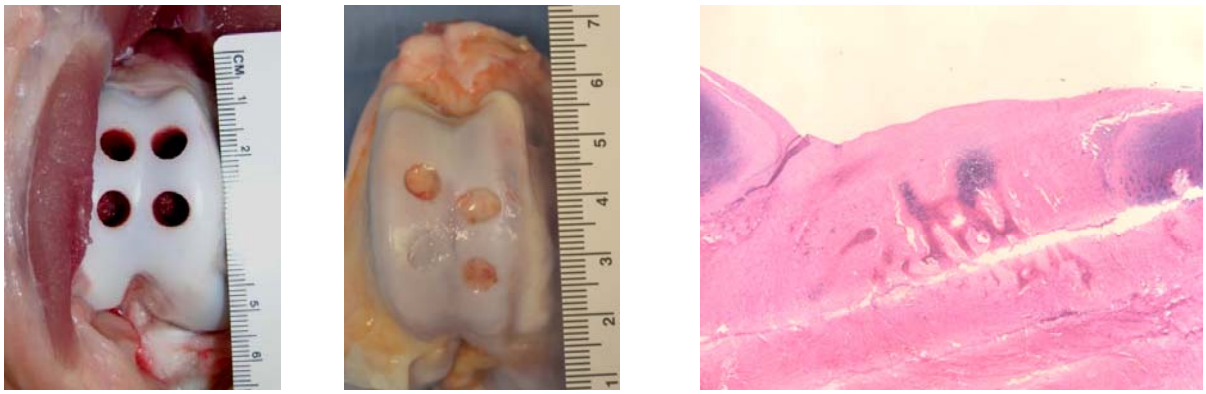


Figure 10. Intraoperative photograph from a swine showing four, 6.5 mm defects made in the trochlear groove of the distal femur (left). Photograph taken at the time of harvest showing that the defects are filled with new tissue (middle). Histology of the tissue, however, demonstrated only minimal amount of new cartilage formation and demonstrated by the blue stained area in the middle of the image (right; H&E).

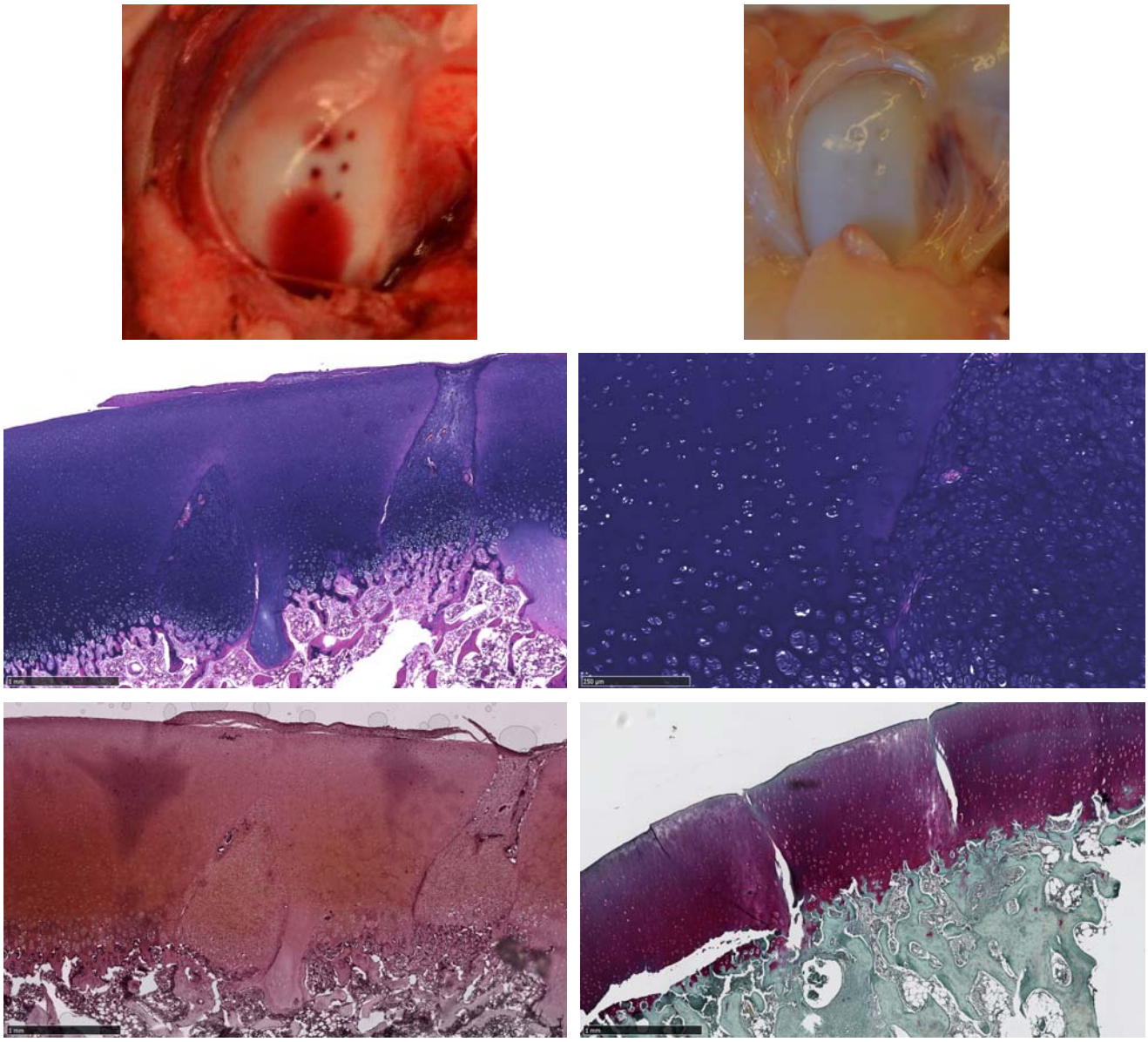
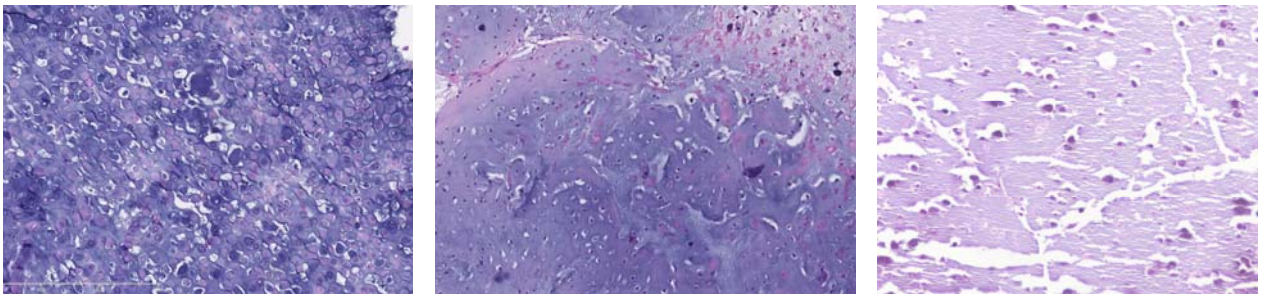


Figure 11. Intraoperative photograph of pig #21454 showing the site of the mini punches that were treated with cells in collagen gel (top left). Photograph of the harvest of pig #21454 where the mini punches were made showing filling of the defects with new tissue (top right). Histology from pig #21454 showing cross sections through two of the punch sites showing the defects were filled with new cartilage matrix (H&E; 1mm scale bar; middle left). Higher magnification of the interface between the neocartilage and the existing native cartilage in pig #21454 (H&E; 250um scale bar; middle right). Safranin O staining demonstrated the presence of GAG in the neocartilage in pig #21454 (Safranin-O; 1mm scale bar; bottom left). Section from pig #21352 that was not treated with cells showing clefts in the articular cartilage where the mini punch defects were made (Safranin-O; 1mm scale bar; bottom right).



chondrocytes **differentiated MSCs** **undifferentiated MSCs**

Figure 12. Swine chondrocytes (left) and MSCs (middle and right) were placed into alginate gels; cultured in vorto for 2 weeks, then placed in vivo into mice for 5 weeks. During the in vitro culture the gels containing MSCs were grown with or without chondrogenic factors provided by commercial Invitrogen mixture. MSCs exposed to the chondrogenic factors showed catrilage matrix formation (middle). Those gels with MSCs not exposed to the growth factors did not demonstrated matrix formation (right).